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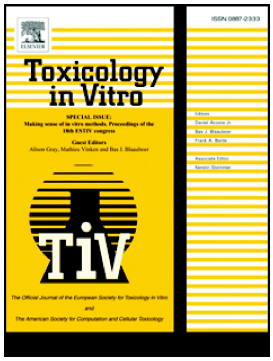

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Effects of copper oxide nanoparticles and copper ions to Zebrafish (*Danio rerio*) Cells, Embryos and Fry

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Abstract

The use of engineered metal nanoparticles (NPs) is continuously increasing and so is the need for information regarding their toxicity. This study compares the toxicity of CuO NPs with ionic Cu in three zebrafish model systems; zebrafish hepatoma cell line (ZFL), fish embryo toxicity test (FET) and fry locomotion. In the ZFL tests, no significant cytotoxicity (cell death, decreased metabolic or cell membrane integrity) was detected for either treatment, though both significantly affected reactive oxygen species (ROS) production. Embryo mortality was affected by both Cu ions and CuO NPs with similar concentration-response relationships, whereas only Cu ions affected fry mortality (24 h LC_{50} ≈30 µM, ≈2 mg Cu L^{-1} for Cu ions and no significant mortality observed at up to 200 µM, 12.7 mg Cu L^{-1} for CuO NP). Both Cu forms increased fry swimming activity during light cycles and decreased activity during dark cycles: Cu ions had significant impact at lower concentrations than CuO NPs. The implications are that Cu ions generally are more toxic than CuO NPs to embryos and fry but there is a marked difference in toxicity among the different zebrafish model systems. Metal NPs release into the environment may have adverse effects on fish and other aquatic organisms.

Keywords: biological organization; cell-line; CuO; embryo; metal; cytotoxicity.

1. Introduction

Nanoparticles (NPs) are increasingly being used in industry and consumer products due to their unique chemical and physical properties caused by their small size (1-100 nm) and high surface to volume ratio (Nel et al. 2006). Though the scientific body of evidence regarding adverse effects of metal NPs using standard toxicity tests is increasing, the information on mechanisms of toxicity is still limited. Therefore, it is important to examine toxicity of these particles and understand the molecular mechanisms explaining possible effects. Previous studies have shown that bioavailability and toxicity of metals and metal NPs may be influenced by species, cell type and level of organization (cell, embryo, juvenile, adult) (Rainbow 2002, Griffitt et al. 2007, Shaw and Handy 2011, Shaligram and Campbell 2013). It is therefore necessary to examine possible negative effects of NP exposure on different levels of biological organization (cells and individuals) and developmental stages (embryos and fry) on toxicity.

The toxicities of a wide array of metal ions to fish and cell cultures are relatively well known compared to that of most metal NPs. For pelagic species (including fish) there is consensus that toxicity of aqueous metal often can be predicted by bioavailability of the free metal ion (Luoma 1983, Flemming and Trevors 1989, Meador 1991, Allen and Hansen 1996). However, metal NPs may have inherent particle-specific effects causing toxicity, in addition to dissolved ions in suspension (Shaw and Handy 2011). For example, Griffitt et al. (2007; 2009) reported that Cu NPs increased gill filament width in zebrafish and resulted in distinct gene expression profile compared to $CuSO_4$. In addition, particles may behave differently in suspension, the mechanisms of uptake may differ (i.e. particle uptake via endocytosis vs uptake of ions via passive or active transport across the cell membrane), and the physical interactions between the particle and the cell may differ from that of dissolved ions. Metal and metal oxide nanoparticles have been hypothesized to promote cytotoxicity and apoptosis potentially via reactive oxygen species (ROS) generation and DNA damage (Misra et al. 2014, Sun et al. 2012, Xu et al. 2012, Thit et al. 2013 and Thit et al. 2015a). Excessive ROS generation can lead to oxidation of proteins inactivating enzyme activity and receptor functions. Oxidation of membrane lipids can cause a radical chain reaction, leading to the formation of peroxides (lipid peroxidation), which can severely damage the cell membrane (Halliwell 1987).

The small tropical freshwater fish, zebrafish (*Danio rerio*) is a widely used model organisms due to its small size, ease of culture, transparency of the embryo and early developmental stages, that allows observations of embryonic development, mortality etc. (Walker et al. 2012, Dai et al. 2014). In addition, this species allows for testing effects at different levels of biological organization and developmental stages: e.g., cells in culture, embryos and fry. Cellular level responses to stressors, such as metals occur prior to effects at organism level and may indicate disturbance of homeostasis and thus the physiological status of the organisms and/or provide information on mechanisms of toxic action of the stressor. Alterations in animal behaviour integrate effects of contaminants in an organism and may reflect a series of toxic events and compensatory responses. Changes in swimming behaviour of exposed fish often occurs at much lower exposure concentrations than mortality and may result in severe ecological consequences (e.g., altered predation, predator avoidance and social behaviour) (Scott and Sloman 2004; Ašmonaitė et al. 2016). Cu ions may, for example, have effects on neurotransmission, which may cause cumulative effects on behavior. These effects would not be picked up by the ZFL cell studies and it is therefore important to be aware of the advantages and disadvantages of each model system.

Here we examine the toxicity of CuO NPs (6 nm) and Cu ions utilizing three zebrafish-based model systems reflecting different levels of biological organization (cells and individuals) and developmental stages (embryos and fry). The toxicity is examined using zebrafish embryos and fry (lethality, hatchability and locomotion) and the mechanisms of toxicity are examined using zebrafish hepatoma cells (metabolic integrity, cell membrane integrity and ROS production) and zebrafish embryos and fry (ROS production).

2. Materials and methods

2.1 Experimental overview

In a series of tests, CuO NPs were studied using the ionic counterpart (Cu ions) as a reference as previously recommended (Petersen et al. 2014; Selck et al. 2016). Effects of the two Cu forms to zebrafish were examined at different levels of biological organization and developmental stages using three different model systems: a zebrafish hepatoma cell line (ZFL), the zebrafish embryo toxicity test (FET) and zebrafish fry locomotion. In addition, particle dissolution in experimental media was measured to aid elucidating the effect of the dissolved fraction on CuO NP toxicity.

2.2 Experimental chemicals

Two separate batches of CuO NPs (6 nm) were used to avoid excessive aging of the particles in stock suspension during the experimental period. Batch one was used for test with ZFL cells and batch 2 for zebrafish embryos and fry. Both batches of CuO NPs were synthesized in aqueous suspension at Natural History Museum (London, UK) as described in detail in Misra et al. (2014) and Thit et al. (2015b). The CuO suspensions contained monodispersed spherical particles. The primary size of particles was determined by Transmission Electron Microscopy to be about 6 nm (batch 1: 6 nm and batch 2: 5.6 nm), and the zeta potential was determined with Zetasizer (batch 1: 44.05 mV and batch 2: 37.8 mV). Additional method description and characterization data on batch 1 is presented in Pang et al. 2013 and on batch 2 in Thit et al. (2015b). Ionic Cu was administered as solid crystalline copper chloride dihydrate ($Cu(II)Cl_2 \cdot 2H_2O$, from Merck) made in an aqueous suspension in sterile autoclaved MilliQ water.

2.2.1 Fate and behaviour of CuO NP in exposure medium

Suspensions of CuO NP were prepared by diluting the stock suspension (2.33 g Cu L^{-1}) in embryo medium or ZFL cell medium to a concentration of 20 or 200 µM at neutral pH (pH 8 for ZFL cell

medium and 7.6 for fish embryo medium) and reduced pH (pH 6). No sonication of the suspensions was performed. Hydrodynamic diameter (determined by Dynamic Light Scattering, DLS) and stability was examined with zeta-sizer (Malvern, Zetasizer Nano-ZS). In addition, dissolution was studied in order to test whether potential CuO NP toxicity resulted from dissolved ions released from CuO NPs during exposure. CuO NP dissolution in zebrafish embryo medium was measured using two different techniques: ion selective electrode (ISE) and dialysis membranes. Before use, the ISE was pre-soaked in a standard solution of 1000 mg Cu L^{-1} for 2 h according to producer instructions. Meanwhile, a calibration curve was prepared by serial dilution of the stock solution (1000 mg Cu L^{-1}) in test medium providing the concentrations: 100, 10, 1, 0.1 and 0.01 mg Cu L^{-1}. To stabilize the ionic strength of 2 % (v/v) 5 M $NaNO_3$ was added to all standards and samples. The calibration curve was prepared as a three point calibration starting with the lowest concentration and then measuring the 2nd highest and lastly the highest concentration used for the calibration curve. For samples outside the three-point calibration curve, a new calibration curve was prepared with either higher or lower standards to cover the sample. Before measuring with the ISE, the pH of the samples were measured and adjusted to pH 6 with 0.1 M HCl (this was done to minimize interference by complexation). For Cu measurements, samples were measured in intervals of 30 s and repeated three times after the ISE had stabilized thus yielding a measure for the spread of the measuring method (3 analytical replicates). Measurements were performed immediately after suspensions were made (<1 h), after 24 and 48 h, respectively.

For determination of dissolution with dialysis membrane, 2 ml suspension was placed inside the dialysis membrane tube (Float-a-lyzer, Spectrum Laboratories, Inc., US) and submerged in 50 ml clean medium. Immediately after the suspension was made (<1 h), after 24 and 48 h a subsample of 0.2 ml was collected inside (i.e., in the middle of the water column without homogenization) and outside the dialysis membrane. Samples were digested with 1.23 ml 7 M HNO_3 and diluted with 3.75 ml MilliQ water before measurement with ICP- MS (Inductively Coupled Plasma-Mass Spectrometry).

2.3.1 Cell culture

The zebrafish hepatoma cell line (ZFL; CRL-2643) was purchased from LGC (UK). Cells were maintained in falcon T175 culture flasks with 12 ml growth medium at 27 °C in air. The growth medium was made from 9.4g DMEM (Dulbecco´s modified Eagle´s medium), 30 ml Hepes (1 M), 13.9 g L-Glutamate, 3.19 g L-Glutamine, 5 ml Insulin (Human Recombinant, 4 mg ml^{-1}), 4 ml

$NaHCO_3$ (7.5 %), EGF (Human Recombinant), 5 % fetal bovine serum and autoclaved MilliQ water (to a final volume of 2 L). All ingredients for the growth medium were purchased from Invitrogen (GIBCO cell culture systems).

2.3.2 Cytotoxicity assessment

The cytotoxicity was measured with the Alamar blue (AB) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) as described in Schirmer et al. (1997) and Bopp and Lettieri (2008). AB was used as markers for metabolic integrity and CFDA-AM for cell membrane integrity. For assessment of cytotoxicity cells were seeded at a density of 70 000 cells per test well in 96-well black polystyrene micro plates (Fisher Scientific) and incubated for 24 h in 200 µl growth medium. Cells were treated with CuO NP, Cu ions, and, as a positive control, the pro-oxidant, menadione, in a range of concentrations (2, 10, 50, 100 and 200 µM) by adding 100 µl growth medium with toxicant to each test well. After 24 h incubation, exposures were terminated by removing the exposure medium. Incubation media (100 µl) consisting of Trisma buffer (pH 7.5) AB and CFDA-AM, was added to each well and incubated on an orbital shaker in the dark for 30 min. Fluorescence of the two fluorescence probes were measured with a microplate reader (Victor 1420) with excitation and emission wavelengths of 530/590 nm for resazurin (in AB) detection and 485/530 for (Carboxyfluorescein, CF in CFDA-AM) detection.

2.3.3 Cell morphology

Cell morphology was recorded in parallel exposures. Cells were exposed at a concentration of 100 µM Cu following the same procedure as for cytotoxicity assessment, except that the cells were seeded in see-through 96 well plates instead of black plates to allow light microscopy. Cell morphology was observed with an inverted microscope (Nikon Eclipse TS 100) equipped with a Nikon digital camera, and digital image acquisition was performed with Nikon ACT-1 software (ver. 2). Pictures were taken of the midpoint of each well at exposure initiation (0 h) and several times during the continuous exposures (i.e., after 2, 4, 24 h, 4, 5 and 7 days). Four replicate test wells were employed per treatment. The digital images were used to assess the proportion of detached cells. The degree of cell coverage was estimated by calculating the dark area of the picture: living cells appear dark and the substratum in the background light. Detached cells appear as white rounded spheres detached from the substratum and the amount of detached cells was estimated by calculating the white area (rounded detached cells) as described in Thit et al. (2013).

All image analysis was carried out using Image J software (developed at the National institutes of health, Bethesda. MD, USA).

2.3.4 Intracellular generation of Reactive Oxygen Species

Intracellular ROS generation was quantified using the fluorogenic probe 2,7-dichlorodihydrofluorescein diacetate (H_2CDCFA) (Bass et al. 1983, Lebel et al. 1992). The method ustilise that the fluorescence intensity of oxidized probe (DCF) is proportional to the ROS level (Wang and Joseph 1999). Cells were seeded at a density of 70 000 cells per test well in 96-well black polystyrene micro plates (Fisher Scientific) and incubated for 24 h in 200 µl growth medium. Subsequently, cells were washed twice with PBS and loaded with H_2CDCFA (40 µM) in the dark for 30 min. Cells were washed carefully twice with PBS and treated with CuO NP, ionic Cu and menadione in a range of concentrations (2, 10, 50, 100 and 200 µM) by adding 100 µl growth medium with toxicant to each test well. Menadione is a well-known redox-cycling molecule that has been used extensively as a model compound for ROS production. DCF fluorescence was measured with a microplate reader (Victor 1420) with excitation and emission wavelengths of 485 nm and 535 nm, respectively, immediately after exposure initiation and at several time points during exposures for 24 h. Cells where loaded with H_2CDCFA once, since this probe should be trapped inside the cell after being hydrolysed by cellular esterases. Three to four replicate wells were used for each treatment and the experiment was repeated three times in total. The increase in ROS generation was calculated as % increase from time 0 (immediately after incubation) to each census time.

2.4.1 Zebrafish embryos and fry

Fertilized zebrafish eggs were obtained from Sahlgrenska (Gothenburg, Sweden). Adult zebrafish were held at 28°C with 14 h light and 10 h dark in breeding tanks containing 2 breeding pairs each. Fertilized eggs for the fish embryo toxicity test (FET) were collected from 6 breeding tanks in the morning 2-3 h post fertilization (hpf). Collected eggs were washed to remove scales and excrements and kept in embryo medium for approximately 2-3 h. Embryo medium was made by dissolving the following salts in MilliQ water and diluting to a final concentration of: $MgSO_4 \cdot 7H_2O$ 245mg/L, KH_2PO_4 20.5 mg/L, Na_2HPO_4 6 mg/L, $CaCl_2 \cdot 2H_2O$ 145 mg/L, KCl 37.5 mg/L, NaCl 875 mg/L. To obtain hatched fry, fertilized zebrafish eggs were collected and kept at 24±3 °C in embryo medium until hatching.

2.4.2 Zebrafish embryo toxicity (mortality and hatchability)

Exposures were conducted according to OECD guideline 236 with slight modifications (OECD, 2013). Briefly, fertilized zebrafish embryos (< 6 hpf) were inspected for viability, and healthy fertilized embryos at similar developmental stage were collected for exposures. Coagulated or unhealthy embryos identified as white and opaque were discarded. Exposures were initiated by briefly transferring embryos to a petri-dish containing clean medium (controls) or exposure solution. Subsequently, the embryos were transferred individually to wells of U-bottom 96-well plates and incubated for 96 h in 250 µl solution. The tested concentrations for each of the two Cu forms were 0.1, 0.5, 2, 10, 50 and 200 µM Cu. During the 5 day exposure, observations were conducted to assess mortality and hatching. Embryo mortality was recorded after 24, 48, 72, 96 and 120 h incubation. Mortality was expressed as percentage of dead embryos at each time point. Hatching success was expressed as percentage of hatched embryos after 72 h. The test was carried out with four replicates per treatment; each consisting of 8 individually exposed embryos or 16 for controls (in total 384 embryos). For each replicate, embryos from 3 separate tanks were used.

2.4.3 Zebrafish locomotion and survival

To study the effects of CuO NPs and Cu ions on zebrafish locomotion (swimming activity), a View Point® automatic behaviour tracking system (Zebralab version 3.22, ViewPoint Life Science Inc., Montreal, CN) was used (Ašmonaitė et al. 2016). This comprises an automated analysis chamber, which monitor the movements of individual zebrafish by capturing 25 images per second. Zebrafish fry (4 days post hatching) were exposed individually in wells of 48-well plates and monitored after 24 and 48 h, respectively, according to the established protocol presented in Ašmonaitė et al. (2016): 18 dark-light cycles (5 min dark + 5 min light) for 1.5 h. Movement was recorded and divided into three categories by the software. 1: large continuous movements in particular direction (distance>6.1 mm), 2: small non-ambulatory movements (distance: 2.1-6.1 mm), and 3: inactivity, minor movements (distance: 0-2.1 mm). These locomotory categories were set according to Viewpoint recommendations. After data collection the total distance travelled was summed up for each category to allow statistical analysis as previously described by Ašmonaitė et al. (2016; figure 5 therein). Dead, completely inactive individuals were excluded from analysis. In addition, zebrafish fry survival was recorded at two time points (i.e., after 24 and 48 h of exposure, respectively).

2.5 Data analysis

Each experiment with ZFL cells was conducted three times, and, for each experiment, the mean (± SD) of thee-four replicates per treatment (except for controls were up to 24 replicates were conducted) was deduced. Each experiment with zebrafish embryos or fry was conducted using 4-8 test plates: 4 individuals exposed to each treatment per plate, except for controls were 8 individuals were used per plate. Individuals exposed in the same test plate were considered one replicate. Results are presented as mean ± SD for each test plate.

Data were analysed using one-way analysis of variance (ANOVA) if the assumptions for ANOVA were met: i.e., data or log-transformed data were normally distributed with equal variances. ANOVA was conducted for metabolic integrity of cells treated with Cu ions and menadione, cell membrane integrity for all treatments, 72 h embryo hatchability, zebrafish fry behaviour (all comparisons of distance travelled except, distance travelled after 24 h in minor movements during light). Tukey's Honestly-Significant-Difference Test was conducted where significant differences among treatments were detected. If data did not meet the assumptions for ANOVA a non-parametric Kruskal-Wallis test was conducted (i.e.: metabolic integrity of cells treated with CuO NPs, proportion of detached cells, intracellular ROS-production after 24h, embryo and fry mortality after 24 h, distance travelled after 24 h in minor movements during light and in minor movements after 48h during dark). If significant differences were detected a Conover-Inman test for all pairwise comparisons was conducted. All data were analysed using SYSTAT 13 software and plots where made using the software Sigmaplot 12.0 (Chicago, IL, USA). A significance level of $p \leq 0.05$ was employed and $0.05 \leq p \leq 0.10$ was considered marginally significant.

3. Results

3.1 Fate and behaviour of CuO NPs in exposure medium

Hydrodynamic diameter and stability of CuO NPs in ZFL medium and zebrafish embryo medium

The hydrodynamic diameter (z-average) of CuO NPs changed with CuO NP concentration, exposure medium, pH and time after the suspension was made. After 48 h incubation in ZFL cell medium, z-averages were between 117 and 163 nm at all scenarios (20 and 200 µM at pH 8 and 6), except one (200 µM at pH 8), where the z-average was 349±101 nm. An initial test adding CuO NP to zebrafish embryo medium showed, that the suspension gave insufficient signal due to sedimentation of CuO NPs after 48 h of incubation (figure S1). The dynamic change in z-average over time was therefore measured and showed an increase in z-average from initially around 1000 nm (t 0) to approximately 1900 nm after 5 h. The signal was insufficient to obtain reliable results

using DLS after approximately 7 h, and was undetectable after 13 h, where after the experiment was terminated. The halftime of NPs in suspension was approximately 2.5 h. Thus, the actual concentrations that the embryos were exposed to are not expected to be constant over time. In addition, agglomeration/aggregation of CuO NPs will likely result in larger CuO NP agglomerates and aggregates found at the bottom of the exposure containers.

Dissolution of CuO NPs in ZFL medium and zebrafish embryo medium

In ZFL cell medium less than 10 % of the original CuO NP load added was detected as dissolved Cu as measured by the ISE after 48 h incubation (figure 1; figure S2). In zebrafish embryo medium dissolution of CuO NPs was even lower than in ZFL cell culture medium as less than 1 % of the added load was detected (ISE) as dissolved Cu (t= 0, 24, 48 h after incubation) (figure 1; figure S2). Also, no dissolved Cu was detected using dialysis membranes. The initial total Cu concentrations inside the dialysis membrane tube were close to the nominal Cu concentrations of 20 and 200 µM (20.5±0.16 and 206.1±0.8 µM, respectively). The concentration inside the dialysis membrane tube decreased to less than 50 % in all samples after 48 h (data not shown), indicating sedimentation in the dialysis tube. No dissolved Cu was detected outside the dialysis membrane tube at all times (up to 48 h of incubation).

3.2 ZFL cells

3.2.1 Cytotoxicity: Metabolic and cell membrane integrity

Changes in metabolic- and cell membrane integrity were normalized by calculating the decrease in treated cells compared to controls (i.e., set to zero) (figure 2, non-normalized data is presented in figure S3) (Schirmer et al., 1997; Bopp amd Lettieri, 2008). Generally, treatment with the pro-oxidant menadione (positive control) resulted in a concentration-response relationship: significant differences in metabolic integrity, p=0.037 (ANOVA), and marginally significant differences in cell membrane integrity, p=0.057, at different concentrations. Though up to 20 % decrease was observed in both metabolic and membrane integrity at higher exposure concentrations (50-200 µM) these differences were not significantly different. No significant differences in cell membrane integrity or metabolic integrity was detected among Cu concentration (both Cu ions and CuO NPs) either (all p-values >0.1, ANOVA for all comparisons except for metabolic integrity of cells treated with CuO NPs where a Kruskal-Wallis test was conducted) (Figure 2). This lack of significance

may be a result of the great variance on data, as illustrated by the large error bars as discussed in section 4.1.

3.2.2 Changes in cell morphology and detachment from substratum

Microscopy images of ZFL cells showed that untreated cells formed a confluent cell layer. Healthy cells were attached to the substratum forming a cell layer (appear dark grey on images), with very few rounded cells detached from the cell layer (figure 3; For controls the ratio between attached and detached cells where close to the ratios usually observed around 0.01 after 5 days of incubation in clean medium). Menadione treatment (positive control, 100 µM) resulted in clear morphological changes, with all the cells rounded and detached from the substratum already 2 h after the treatment was initiated (figure 3, top and figure S4). Menadione treatment caused a significant increase in the proportion of detached cells with time (non-parametric Kruskal Wallis; p=0.039, figure S4). Neither ionic Cu nor CuO NP treatment resulted in any clearly visible changes to cellular morphology (figure 3). The proportion covered by detached cells increased significantly with time for both treatments (non-parametric Kruskal Wallis; p=0.038 and 0.028 for Cu ions and CuO NPs, respectively; figure S4). However, there were no significant difference in the proportion of detached cells between Cu treatments or compared to the control, except marginally significant differences between Cu ions and control and Cu ions and CuO NPs after 96h (p= 0.083 and 0.053, respectively). Overall, there were a pronounced effect of menadione and very little effects of ionic Cu or CuO NPs on cell morphology and cell detachment.

3.2.3 Intracellular generation of reactive oxygen species

ROS production from untreated ZFL cells increased by 190.3±37 % during 24 h (figure 4). Menadione increased fluorescence from cells in a concentration-dependant manner with a maximum increase in fluorescence of 783.3±149 % in the 200 µM treatment. The increase in ROS production after 24 h treatment with menadione was significantly higher than controls (2 µM p= 0.013, 10 µM p=0.043, 50, 100 and 200 µM p=0.000) (figure 4). Ionic Cu caused a concentration-dependent increase in ROS production with a maximum increase of 334.3±40 % at 200 µM after 24 h (figure 4b), which was significantly different from control at 50, 100 and 200 µM (p=0.002, 0.008 and 0.000, respectively). Treatment with CuO NPs also resulted in a significantly higher increase in fluorescence compared to the control at 100 µM (255.6±36 %; p=0.013). At 200 µM the increase in ROS production was 216.2±44 %, however, this increase was not significantly different from the

control (figure 4). Comparison among treatments after 24 h, showed that ionic Cu caused a significantly higher increase in florescence compared to CuO NPs at 10, 50 and 200 µM (p= 0.043, 0.008 and 0.003, respectively, figure 4).

3.3 Zebrafish embryos and fry

3.3.1 Zebrafish embryo mortality

The concentration-response relationships for the two Cu treatments overlapped (LC_{50}s were estimated to approximately 10 µM for both treatments; figure 5). However, the LC_{50} values are based on few data points and are thus only rough estimates illustrating that the toxicity of the two treatments are relatively similar. Both Cu treatments resulted in significantly increased embryo mortality compared to the control, at 50 and 200 µM (all p-values below 0.05). There were no significant differences between the two Cu treatments at any of the tested concentrations (all P>0.10). No mortality occurred in controls after 24 h incubation. For embryos treated with Cu ions or CuO NPs (figure S5) the concentration-response relationships for the latter time points (after >24 h exposure) were almost identical to the one for 24 h exposure, and therefore the concentration-response relationship is only shown for 24 h exposure.

3.3.2 Hatchability

In general, zebrafish embryo hatchability decreased with increasing concentrations for both Cu treatments (figure 5). Hatchability of embryos treated with Cu ions was significantly lower than in the control at 0.5 µM (p= 0.039), 2 µM (p= 0.001), 10 µM (p= 0.000) and 50 µM (p= 0.003). For CuO NPs, hatching was significantly different from the control at 0.1 µM (p=0.031), 10 µM (p= 0.000) and 50 µM (p= 0.000). Cu ions showed greater effects on hatching than CuO NPs and at 2 µM significantly fewer embryos hatched during 24 h incubation with Cu ions (<20 % hatched) compared to CuO NPs (≈60 % hatched) (p< 0.001). The hatching at 200 µM is not presented since there were no surviving embryos at this concentration.

3.3.3 Zebrafish fry mortality

There was no observed mortality of zebrafish fry exposed to either Cu-form at the lower concentrations (i.e., 0-10 µM). Significant increase in mortality was observed for fry exposed to Cu ions at the two highest concentrations (50 and 200 µM) compared to the control (both p< 0.001). Mortality of fry exposed to Cu ions at these concentrations was also significantly higher than for fry exposed to CuO NPs (figure 6), (p= 0.000 for both pairwise comparisons). LC_{50} was approximately

30 µM for the Cu ion treatment, which equals ≈2 mg Cu L^{-1}. Since, mortality was low (< 10 %) for fry in the CuO NP treatment, the LC_{50} was estimated to be above the highest tested concentration (i.e. LC_{50} > 200 µM (12.7 mg Cu L^{-1})).

3.3.4 Zebrafish swimming activity

Control fry were very active during the dark cycles and much less active during light cycles (figure S6; figure S7; table 2) with longer total distances travelled in large movements (i.e. movements >6.1 mm) during dark cycles compared to during light-cycles. For example, the total mean distance travelled (i.e., total during all cycles) by control fry in large movements was about 4500 mm during dark cycles and below 1000 mm during light cycles after both 24 and 48 h (figure 7 and 8). Similarly, the distance travelled in small movements (between 2.1 and 6.1 mm) was longer during dark cycles (above 5000 mm travelled in total) than during light cycles (about 3000 mm in total distance travelled), whereas the distance travelled in minor movements during light and dark cycles were similar (both about 1000 mm in total) after 24 and 48 h incubation (figure 7 and 8).

Effects of Cu ions on zebrafish fry swimming activity

After 24 h incubation with Cu ions at 2 µM the distance travelled in large movements during dark (figure 7a) was significantly decreased compared to control (p=0.035). During light, the total distance travelled in both large (> 6.1 mm) and small movements (2.1-6.1 mm) increased significantly by exposures to 2 µM Cu ions (figure 7b, d) (p= 0.003 and p= 0.023, respectively). Inactivity (i.e., minor movements; < 2.1 mm) increased significantly by exposure at 2 and 10 µM compared to controls both during dark (figure 7e; p= 0.043 for both comparisons) and light cycles (p= 0.002 and 0.001, respectively). After 48 h incubation, there was no significant differences between Cu exposure (both treatments) and control in the total distance travelled in large movements. After incubation with Cu ions at 10 µM, the distance travelled in small movements during dark was significantly decreased compared to controls (p= 0.033), whereas there were no significant differences during light cycles. There were no significant differences in total distance travelled in minor movements during dark or light cycles. No statistics were conducted for fry exposed to Cu ions at 50 µM and 200 µM as only one or no individuals survived 48 h of exposure, respectively.

Effects of CuO NPs on zebrafish fry swimming activity

Fry exposed to 10 µM CuO NPs were almost equally active during light and dark cycles after both 24 and 48 h (figure S6; figure S7; figure 7 and 8). There were no significant differences in total distance travelled (in large, small, or minor movements, respectively) by fry exposed to CuO NPs and control fry during dark cycles after either 24 or 48 h exposure. However, after 24 h exposure to CuO NPs at 10 µM the total distance travelled in large, small and minor movements during light cycles was significantly higher than for controls (figure 7b, d, f p= 0.000, 0.036 and 0.013, respectively). After 48 h of exposure to CuO NPs there were no significant differences in distance travelled for any of the categories (large, small or minor movements) compared to controls at any of the tested exposure concentrations during either light or dark cycles.

4. Discussion

4.1 Does toxicity of CuO NPs and Cu ions differ among levels of biological organization and developmental stages?

Toxicity differed greatly among levels of biological organization (cells vs individuals) and developmental stages (embryos vs fry) (see Table 2). Generally, toxicity of Cu ions was higher than for CuO NPs for embryos and especially fry, whereas limited effects was observed for either Cu treatments on ZFL cells. This aligns well with previous findings of a higher resistance of ZFL cells than zebrafish fry (Chen et al. 2011). These authors reported 96 h LC_{50s} for Cu_2O NPs of 1545 µM for ZFL cells and 3.39 µM for zebrafish fry. Limited detection of toxic effects to ZFL cells does not necessarily mean that there are no toxic effects, but simply that no effects were detected in the present study with the methods used. The great error bars seen in figure 2 illustrates the pronounced variation on cytotoxicity measured as cell membrane and metabolic integrity. However, the large increase in cytotoxicity after treatment with menadione are detectable with the assay, in spite of the great variation. In general, the published literature shows different results with varying model systems and exposure conditions. Some studies using aquatic organisms have shown that toxicity of metal NPs is lower than for their corresponding metal ions to Daphnia, algae, adult zebrafish and frog embryos (Griffitt et al. 2007, Navarro et al. 2008, Kennedy et al. 2010, Bacchetta et al. 2012, Thit et al. 2017). In contrast, higher toxicity of CuO NPs compared to Cu salt have been reported for a range of cell lines (Shaligram and Campbell, 2013), *in vitro* studies with kidney and hepatoma cells, and *in vivo* studies with juvenile zebrafish. The authors argued it was unlikely that dissolved ions alone could explain the toxicity of metal NPs (Griffitt et al. 2008, Kawata et al. 2009, Thit et al. 2013). Furthermore, as stated in Petersen et al. (2014), it is important to consider that different

test media and exposure setups will impact the state of agglomeration and dissolution of NPs. Accordingly, the DLS and ISE data showed that the pattern of agglomeration and dissolution was substantially different between ZFL cell medium and zebrafish embryo medium. Consequently, the routes of exposure may differ, thus leading to different mechanisms of uptake and sites of action for NP compared to soluble compounds. Our findings underline the importance of considering the level of biological organization, developmental stage as well as exposure scenario when assessing ecotoxicity of metal NPs, especially before drawing generalizing conclusions. In addition it is important to keep the limit of detection for each assay in mind when interpreting toxicity results.

4.2 Can CuO NP toxicity to zebrafish embryos and fry be explained by dissolved Cu ions?

There is an ongoing discussion on whether toxicity of metal NPs is a result of dissolved ions, particle-specific effects or a combination (Navarro et al. 2008, Kawata et al. 2009, Kennedy et al. 2010, Thit et al. 2017). Here we found that toxicity was higher for Cu ions than for CuO NPs towards zebrafish embryos and especially fry. Though the 24 h LC_{50}s for embryos were similar for the two Cu treatments, the significantly higher mortality at 50 µM compared to CuO NPs indicate a higher toxicity of Cu ions. The difference in toxicity was much more pronounced for using fry where the LC_{50} for ions (≈30 µM) was considerably lower than for CuO NPs (> 200 µM). These LC_{50} values are only indicative (and likely lower) since sedimentation of CuO NPs was pronounced resulting in a decreased CuO NP concentration in the water column, increased concentration at the bottom of the exposure containers and adsorption of NPs to embryos as seen on figure 5.

Both Cu treatments affected hatching of embryos, but no developmental malformations were detected after exposure to either of the treatments. Hatching can be delayed by the presence of different metals, such as Cu, by affecting the activity of proteolytic enzymes involved in the breakdown of the embryo chorion (Muller et al. 2015). Thus, it may be hypothesized that dissolved Cu ions released from CuO NPs may cause the reduced hatching of treated embryos. However, even though the free Cu ion concentration is expected to be much lower in the CuO NP treatments than in the Cu ion treatments exposure to Cu ions only resulted in slightly more severe effects than CuO NPs. Thus it seems unlikely that the effects of CuO NP can solely be explained by dissolved ions which is in accordance with the findings of Muller et al. (2015). However, NPs adhered to the chorion may release dissolved ions into the embryo and serve as a direct source of Cu ions.

Fry swimming activity was impacted both by ionic Cu and CuO NPs. However, zebrafish fry activity (distance travelled) was significantly impacted by Cu ions at lower concentrations than

CuO NPs. Similarly, Griffith et al. (2008) exposed aquatic organisms to a variety of metals and metal NPs and reported that dissolved metals in general was more toxic than the corresponding NPs based on metal mass added: Dissolved Cu (administered as $CuSO_4$) was highly acutely toxic (48 h LC_{50}: 0.25 mg Cu L^{-1}) whereas Cu NPs (80 nm primary particle size) was only moderately toxic (48 h LC_{50}: 1.56 mg Cu L^{-1}) to adult zebra fish (Griffitt et al. 2007). However, although toxicity of dissolved Cu was higher than for CuO NPs, the authors argued that dissolved Cu ions could not explain the toxic effects of Cu NPs.

We detected that the embryo medium caused a high agglomeration followed by sedimentation of CuO NP, which may also have decreased the potential for dissolution. Additionally, the availability of single CuO NP to the fry may have been decreased by agglomeration and sedimentation, which could explain the lack of CuO NP toxicity toward zebrafish fry. Griffitt et al. (2007) reported that Cu dissolution from NPs in test water was very low (< 0.1 %) and the authors argued that the toxicity of Cu NPs to adult zebrafish was not solely due to dissolved Cu ions. In addition, Griffitt et al. (2007) found nano-specific effects of Cu nanoparticles on zebrafish gills different from the effects of dissolved Cu. Cu NPs resulted in distinct gene expression patterns and morphological effects in zebrafish gill compared to soluble copper, suggesting that the effects of Cu NPs are not mediated solely by dissolved Cu released from Cu NPs.

4.3 Can CuO NP toxicity to embryos and fry be explained by ROS production?

There is a general belief that toxicity of many metal NPs, including CuO NPs, is a result of ROS production. Our ZFL cell studies showed that both treatments (Cu ions and CuO NPs) resulted in an increase in ROS over the incubation period (from initiation, t 0), which was significantly different from controls at ≥50 µM for Cu ions, but only at 100 µM for CuO NPs. The lacking significant increase in ROS production during majority of the CuO NP treatments are not a result of non-responsive cells, since ROS production was increased by menadione. We did not observe significant increased ROS production in zebrafish embryos or fry exposed to either of the two Cu treatments (data not shown). Therefore, it does not seem likely that the observed toxicity of CuO NPs toward embryos and fry is attributable to ROS-generation. Similarly, Thit et al. (2015a) reported a lack of ROS-production during treatment of kidney cells with CuO NPs in the same size (i.e., 6 nm) as tested here, whereas larger CuO NPs (< 100 nm) increased ROS production. We did not detect toxicity (cell death, decreased metabolic or membrane integrity or ROS production) of

either of the Cu forms tested in the ZFL cell studies. Thus, the tests did not reveal the mechanisms of toxicity of 6 nm CuO NPs toward zebrafish and remains to be elucidated.

4.4 How does CuO NPs affect zebrafish fry?

Behaviour, such as swimming activity, is often impaired at lower concentrations than those causing significant mortality. This was also the case in our study where we did not detect significant increase in mortality of zebrafish fry in response to CuO NP exposure. Swimming activity (distance travelled) was significantly affected by both Cu treatments, such that exposed fry showed unnatural response to light (Cu ions and CuO NPs) and darkness (Cu ions). Zebrafish fry are naturally more active during dark than during light, but CuO NP exposures increased the activity of fry during light cycles. Fish behaviour can be impaired by, for example, disruption of the sensory, hormonal, neurological or metabolic system, where all of these four have been shown to be affected by Cu (reviewed by Scott and Sloman, 2004). This altered behaviour can leave the fish unable to function in an ecological context (Scott and Sloman 2004). Changes in behaviours such as predation, predator avoidance, and social behaviour may have severe ecological consequences not only for the individual, but also on population level.

5 Conclusions

Our study shows that there is a great variation in toxicity of CuO NP (6 nm) and Cu ions to different levels of biological organization and developmental stages of zebrafish. No toxicity of either Cu form was detected in studies with ZFL cells, whereas mortality and decreased hatching was observed for zebrafish embryos. Zebrafish fry mortality was only caused by Cu ions, whereas swimming activity was affected both by Cu ions and CuO NPs (Overview in Table 2). Overall, Cu ions were more toxic than CuO NPs to both embryos and fry. CuO NP toxicity could not solely be explained by dissolution of CuO NPs into exposure medium, since less than 1 % was dissolved during 48 h incubation. The findings indicate that release of CuO NPs into the environment may have consequences for fish and other aquatic species in the environment and that the inevitable release of these particles should be given attention.

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Figure 1: Dissolution of CuO NPs in ZFL cell medium (left) and ZF embryo medium (right) used in exposures at two selected concentrations (20 and 200 µM). Dissolution is presented as percentage of original mass of Cu added present as dissolved Cu. Mean ± SD, n=3.

Figure 2: Effects of Cu ions, CuO NPs and menadione (positive control) on ZFL cells after 24 h treatment at 5 different concentrations. a-c) Relative reduction in metabolic integrity caused by different treatments measured with AB (Alamar Blue). d-f) Relative reduction in cell membrane integrity caused by different treatments measured with DFDA-AM (5-carboxyfluorescein diacetate acetoxymethyl ester). Results are presented as mean ± SD based on 3 replicate experiments (n=3).

Figure 3: Effects on ZFL during treatment with clean medium (control), Ionic Cu, CuO NPs or menadione (positive control) at 100 µM. Top) Effects on ZFL cell morphology and detachment 5 days after exposure initiation. Healthy cells are attached to the substratum and form a dark grey cell layer. Rounded cells detached from the substratum appear as white spheres. Bottom) Effects on ZFL cell detachment during 5 days of treatment with clean medium (control), Ionic Cu or CuO NPs. Proportion of detached cells, calculated as area covered by detached cells compared to area covered by cells attached to the substratum (viable cells) based on digital images, analyzed with ImageJ software, and presented as mean ± SD. n=4.

Figure 4: ROS generation from ZFL cells during 24 h treatment with menadione (positive control), Ionic Cu or CuO NPs at 5 different concentrations. ROS-generation was measured as DCF fluorescence generated from ZFL cells after DCFH-DA incubation for 30 min and presented as mean ± SD based on three experiments (n=3). The increase in ROS generation was calculated as % increase from time 0 (immediately after incubation) to the time of measurement.

Figure 5: Zebrafish embryo mortality and hatchability. Top) Per cent embryo mortality after 24 h exposure to Ionic Cu or CuO NPs at 6 different concentrations. Middle) Percent surviving embryos that have hatched after 72 h exposure to Ionic Cu or CuO NPs at 6 different concentrations. Mortality and hatchability presented as mean ± SD based on 8 replicates. Bottom) from left to right: newly fertilized zebrafish embryo, coagulated embryo exposed to Cu ions at 50 µM for 24 h, developing zebrafish embryo exposed to CuO NP at 50 µM for 24 h, with CuO NP agglomerates/aggregates seen settled on bottom or adhered to embryo (examples are marked with arrows, newly hatched zebrafish fry exposed to CuO NP (2 µM) for 72 h.

Figure 6: Zebra fish fry mortality. Left) Mortality after 24 h exposure to Ionic Cu or CuO NPs at 6 different concentrations presented as mean ± SD of 8 replicates. Right) Zebrafish fry exposed to CuO NPs with agglomerates/aggregates of CuO NPs settled at the bottom of the exposure well marked with arrow.

Figure 7: Distance travelled by zebrafish during 1½ h test after 24 h exposure to Ionic Cu or CuO NPs. Data on position of individual fish fry was gathered every minute and presented as the sum of the total distance travelled during dark periodes (left) and during light periods (right) in large movements (>6.1 mm, top), small movements (2.1-6.1 mm, middle) ansinactivity (minor movements <2.1 mm, buttom). Data is presented as mean ± SD based on 4 replicates. Each replicate consisted of 4 individually exposed fry, or 8 for controls. Significant diffrences (p<0.05) compared to control are marked with *.

Figure 8: Distance travelled by zebrafish during 1½ test after 48 h exposure to Ionic Cu or CuO NPs. Data on position of individual fish fry was gathered every minute and presented as sum total distance travelled during dark periodes (left) and during light periods (right) in large movements (>6.1 mm, top), small movements (2.1-6.1 mm, middle) and inactivity (minor movements <2.1 mm, buttom). Data is presented as mean ± SD based on 4 replicates. Each replicate consisted of 4 individually exposed fry, or 8 for controls. Significant diffrences (p<0.05) compared to control are marked with *.

* *

Tabe 1: Dissolution of CuO NPs in ZFL cell medium and ZF embryo medium used in exposures at two selected concentrations (20 and 200 µM corresponding).

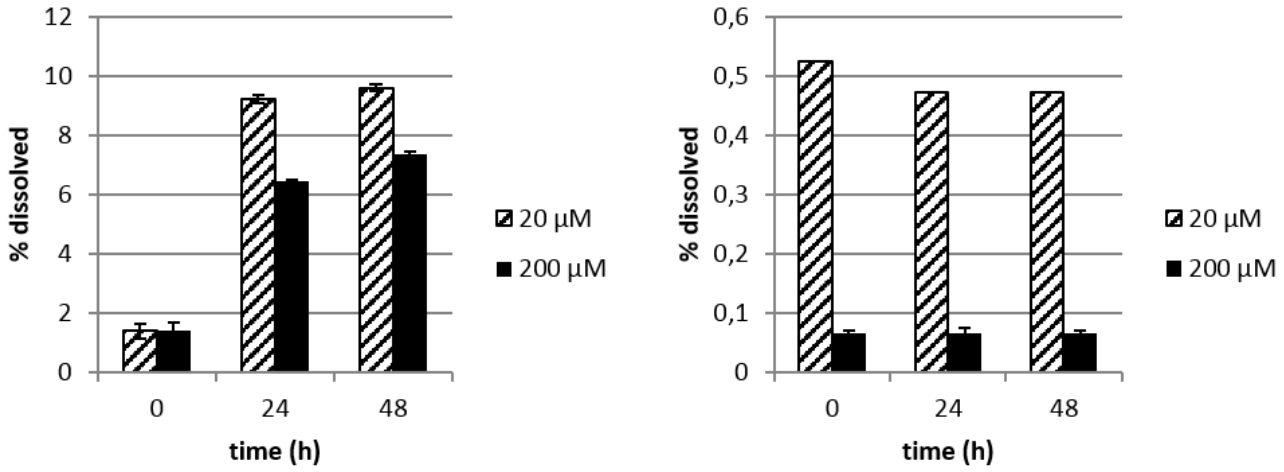
	Cu^{+2} Concentration	0 h	24 h	48 h
ZF cell medium	20µM	0.0176 ± 0.0032	0.1172 ± 0.0018	0.1220 ± 0.0014
	200µM	0.1778 ± 0.0334	0.8172 ± 0.0054	0.9334 ± 0.0126
ZFL embryo medium	20µM	0.0067 ± $1x10^{-18}$	0.0060 ± $1x10^{-18}$	0.0060 ± $1x10^{-18}$
	200µM	0.0083 ± 0.0006	0.0083 ± 0.0012	0.0083 ± 0.0006

Dissolution is presented as total concentration of Cu^{2+} (mg L^{-1}) measured in suspension. Presented as mean ± SD based on 3 analytical replicates.

Table 2: Comparison between toxicity of Cu ions and CuO NPs at different levels of biological organization.

Model system	Highest effect			
	Ions > NP: Ions have significantly higher effects than NPs	Ions ≈ NP: Both Cu treatments cause effects at a similar level	Ions/NP: No statistically significant effects of either Cu treatments	NP > Ions: NPs have significantly higher effects than ions
ZFL cells	***1) ROS generation (24 h):*** Both treatments increased ROS generation. Ionic Cu caused a significantly higher increase compared to CuO NPs at 10, 50 and 200 µM.		***1) Metabolic integrity:*** No significant difference between treatments. ***2) Membrane integrity:*** No significant difference between treatments. ***3) Cell detachment:*** Significant increase over time (4 h, 1, 4, 5 d) for both treatments, but no significant difference between Cu treatments or compared to the control.	
ZFL embryo	***1) Hatchability:*** Both Cu treatments resulted in decreased hatching. 2 µM Cu ions significantly decreased number of embryos hatched during 24 h incubation compared to CuO NPs.	***1) Mortality:*** Both Cu treatments significantly increased embryo mortality compared to the control, at 50 and 200 µM. There were no significant differences between the two Cu treatments at any of the tested concentrations.	***1) ROS generation:*** No significant difference between treatments.	
ZFL fry	***1) Swimming activity in dark (24 h):*** Cu ions at 2 µM decreased the distance travelled in large movements. Cu ions at 2 and 10 µM increased Inactivity. ***2) Swimming activity in dark (48 h):*** Cu ions at 10 µM decreased the distance travelled in	***1) Swimming activity in light (24 h):*** 2 µM Cu ions and 10µM CuO NPs increased distance travelled in both large and small movements. 2 and 10 µM Cu ions and 10 µM CuO NPs increased Inactivity.	***1) Swimming activity in light (48 h):*** No significant differences between treatments and control at any concentration.	

	small movements, whereas there was no significant effects of CuO NPs at any concentrations. ***3) Mortality:*** Mortality of fry exposed to Cu ions at 50 and 200 µM was significantly higher than for fry exposed to CuO NPs.	



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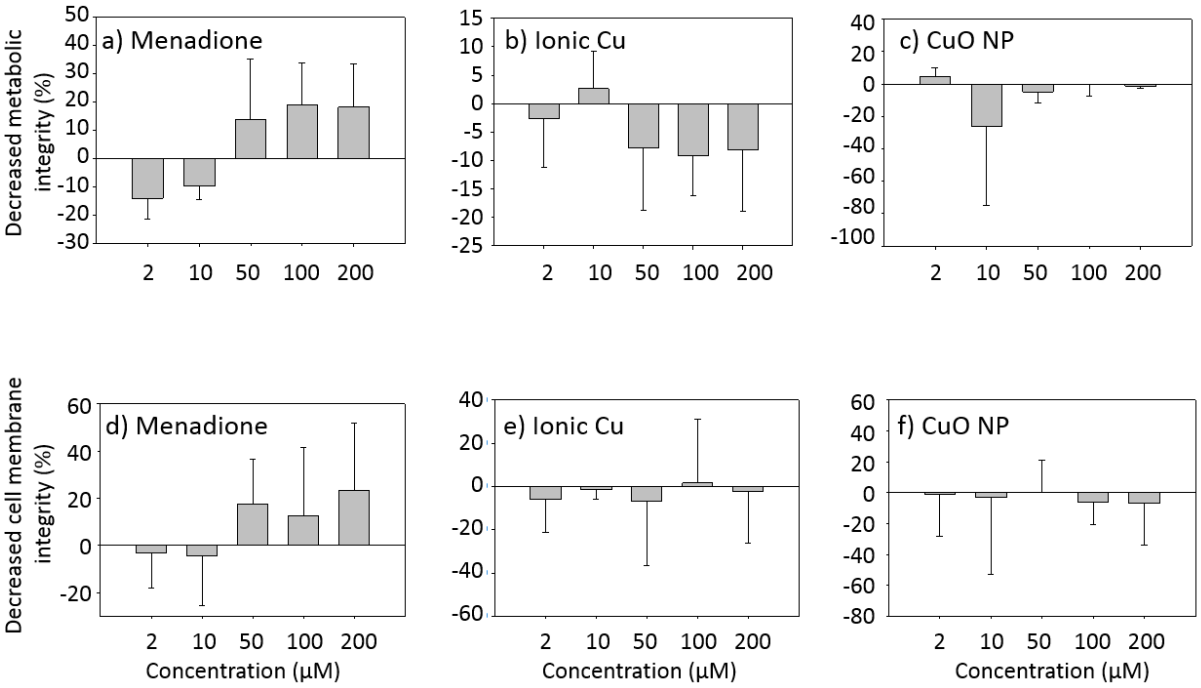


Fig. 2

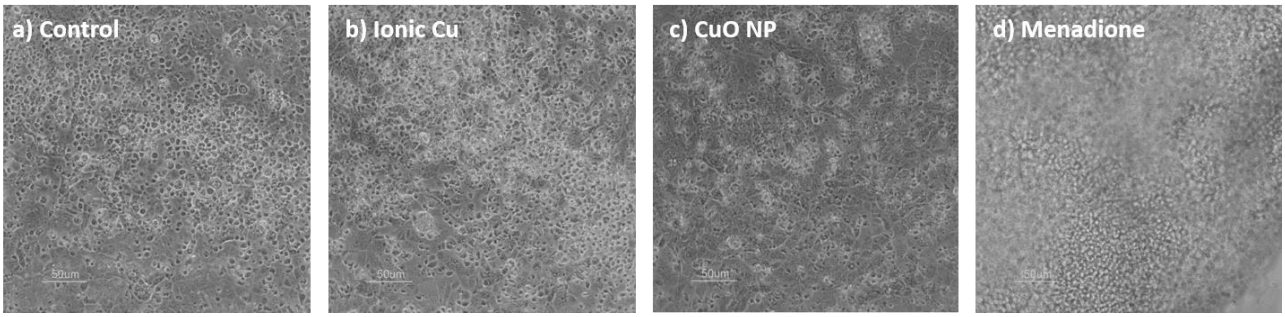


Fig. 3 top

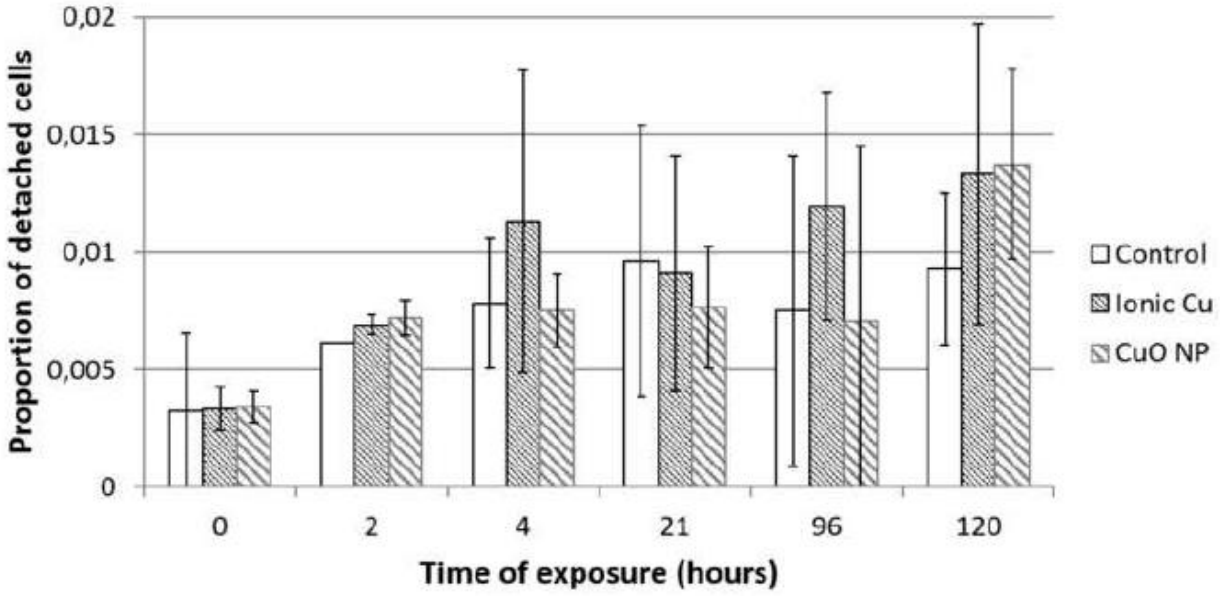


Fig. 3b

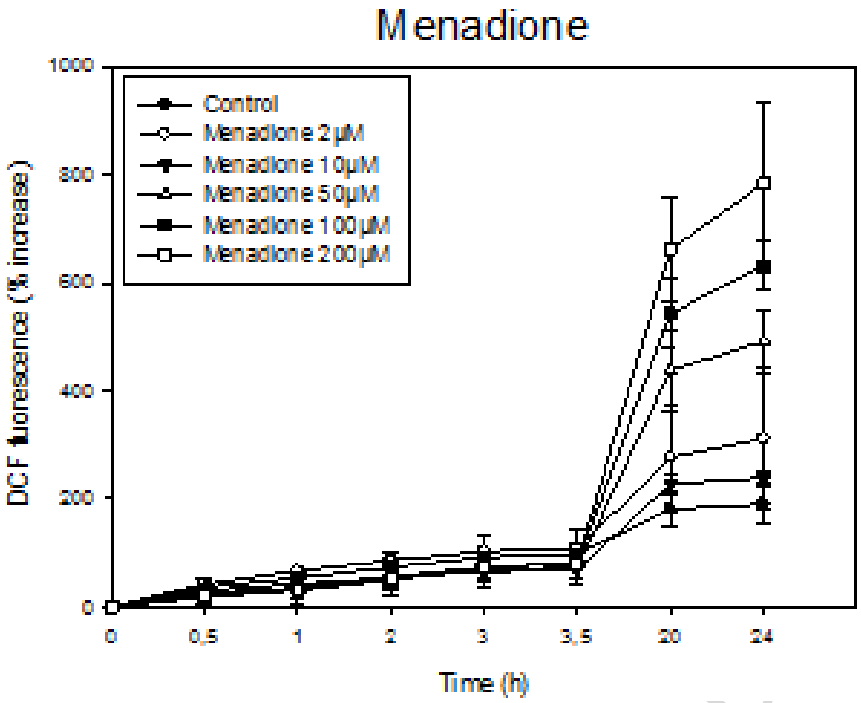


Fig. 4a

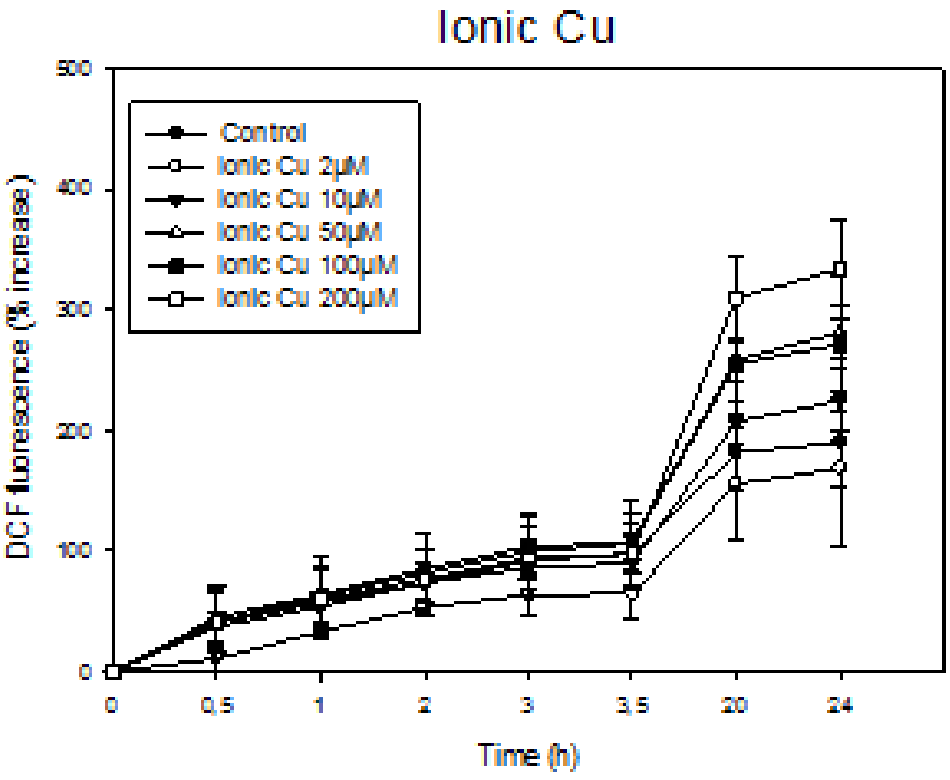


Fig. 4b

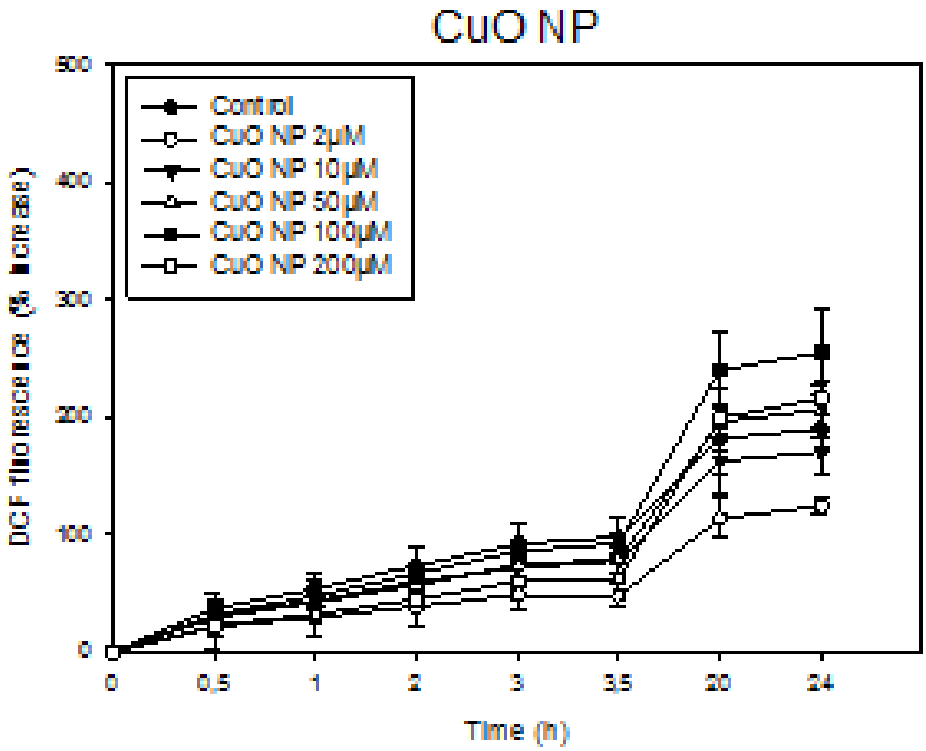
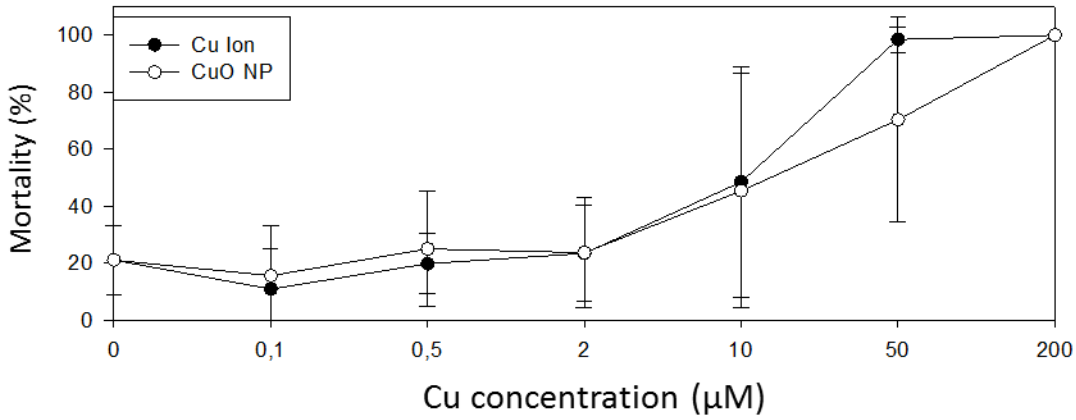
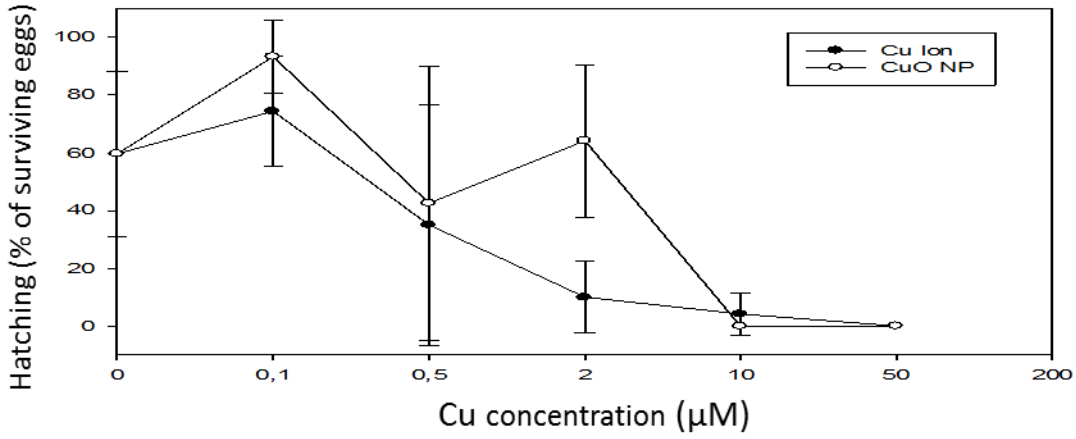


Fig. 4c





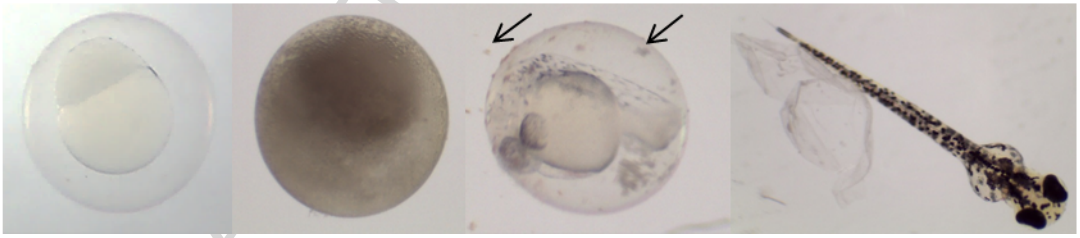


Fig. 5

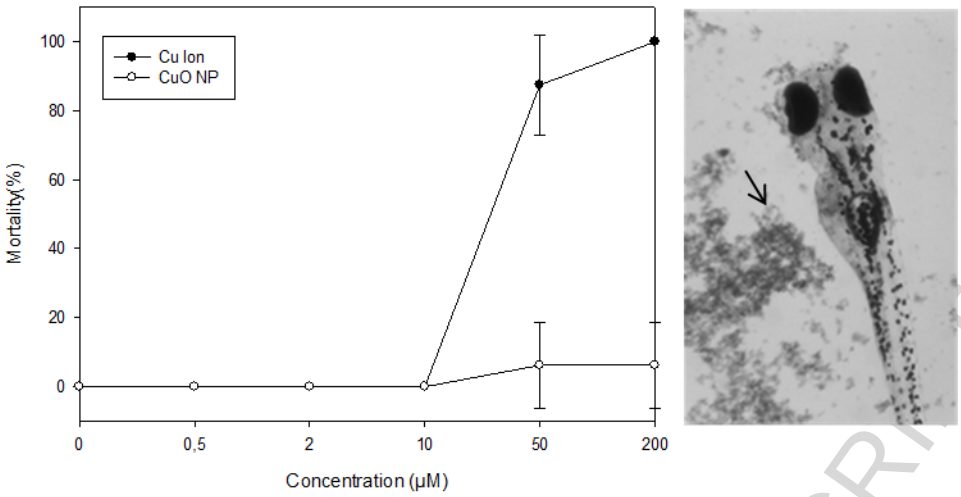


Fig. 6

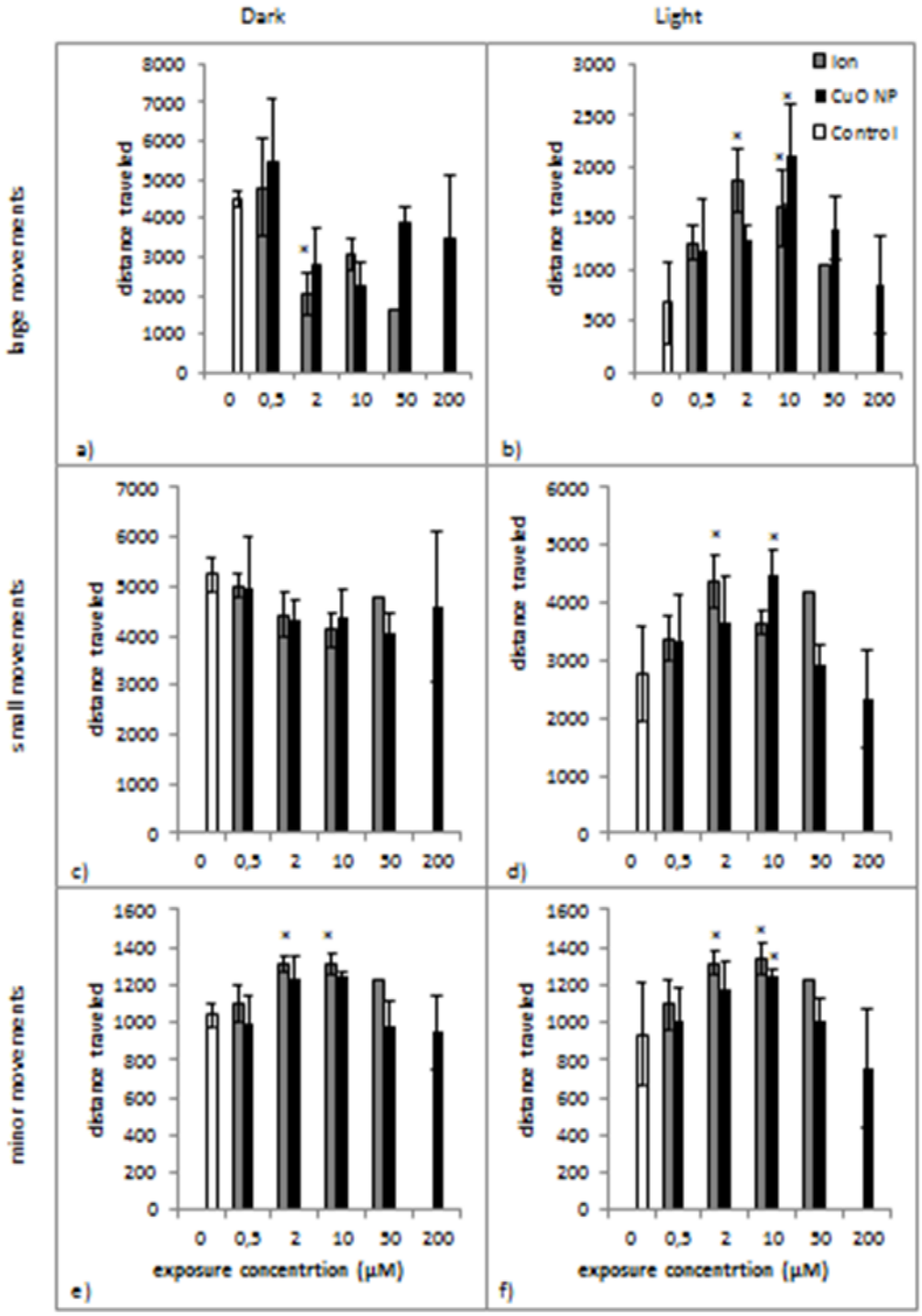


Fig. 7

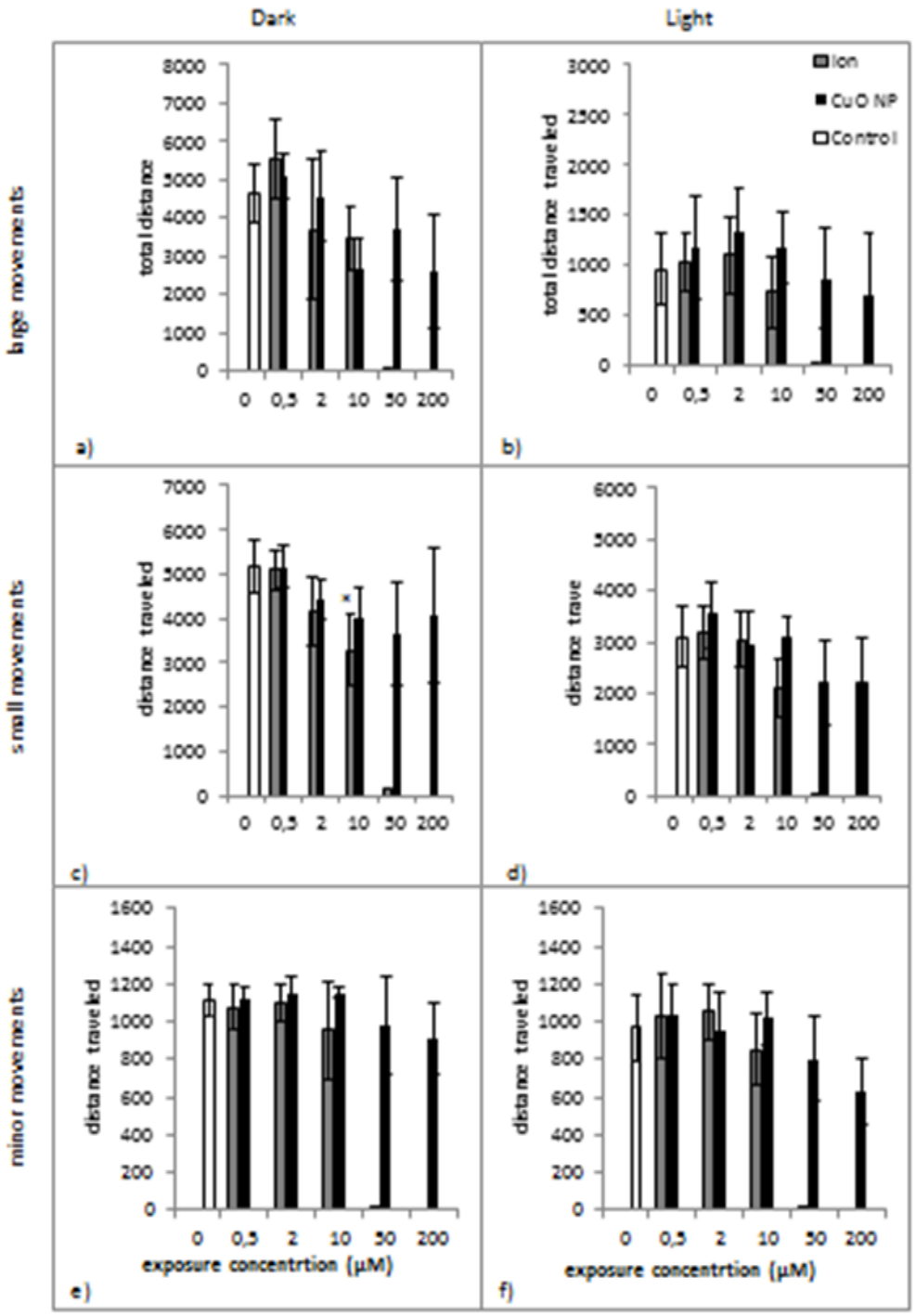


Fig. 8

Figure 1: Dissolution of CuO NPs in ZFL cell medium (left) and ZF embryo medium (right) used in exposures at two selected concentrations (20 and 200 µM). Dissolution is presented as percentage of original mass of Cu added present as dissolved Cu. Mean ± SD, n=3.

Figure 2: Effects of Cu ions, CuO NPs and menadione (positive control) on ZFL cells after 24 h treatment at 5 different concentrations. a-c) Relative reduction in metabolic integrity caused by different treatments measured with AB (Alamar Blue). d-f) Relative reduction in cell membrane integrity caused by different treatments measured with DFDA-AM (5-carboxyfluorescein diacetate acetoxymethyl ester). Results are presented as mean ± SD based on 3 replicate experiments (n=3).

Figure 3: Effects on ZFL during treatment with clean medium (control), Ionic Cu, CuO NPs or menadione (positive control) at 100 µM. Top) Effects on ZFL cell morphology and detachment 5 days after exposure initiation. Healthy cells are attached to the substratum and form a dark grey cell layer. Rounded cells detached from the substratum appear as white spheres. Bottom) Effects on ZFL cell detachment during 5 days of treatment with clean medium (control), Ionic Cu or CuO NPs. Proportion of detached cells, calculated as area covered by detached cells compared to area covered by cells attached to the substratum (viable cells) based on digital images, analyzed with ImageJ software, and presented as mean ± SD. n=4.

Figure 4: ROS generation from ZFL cells during 24 h treatment with menadione (positive control), Ionic Cu or CuO NPs at 5 different concentrations. ROS-generation was measured as DCF fluorescence generated from ZFL cells after DCFH-DA incubation for 30 min and presented as mean ± SD based on three experiments (n=3). The increase in ROS generation was calculated as % increase from time 0 (immediately after incubation) to the time of measurement.

Figure 5: Zebrafish embryo mortality and hatchability. Top) Per cent embryo mortality after 24 h exposure to Ionic Cu or CuO NPs at 6 different concentrations. Middle) Percent surviving embryos that have hatched after 72 h exposure to Ionic Cu or CuO NPs at 6 different concentrations. Mortality and hatchability presented as mean ± SD based on 8 replicates. Bottom) from left to right: newly fertilized zebrafish embryo, coagulated embryo exposed to Cu ions at 50 µM for 24 h, developing zebrafish embryo exposed to CuO NP at 50 µM for 24 h, with CuO NP agglomerates/aggregates seen settled on bottom or adhered to embryo (examples are marked with arrows, newly hatched zebrafish fry exposed to CuO NP (2 µM) for 72 h.

Figure 6: Zebra fish fry mortality. Left) Mortality after 24 h exposure to Ionic Cu or CuO NPs at 6 different concentrations presented as mean ± SD of 8 replicates. Right) Zebrafish fry exposed to CuO NPs with agglomerates/aggregates of CuO NPs settled at the bottom of the exposure well marked with arrow.

Figure 7: Distance travelled by zebrafish during 1½ h test after 24 h exposure to Ionic Cu or CuO NPs. Data on position of individual fish fry was gathered every minute and presented as the sum of the total distance travelled during dark periodes (left) and during light periods (right) in large movements (>6.1 mm, top), small movements (2.1-6.1 mm, middle) ansinactivity (minor movements <2.1 mm, buttom). Data is presented as mean ± SD based on 4 replicates. Each replicate consisted of 4 individually exposed fry, or 8 for controls. Significant diffrences (p<0.05) compared to control are marked with *.

Figure 8: Distance travelled by zebrafish during 1½ test after 48 h exposure to Ionic Cu or CuO NPs. Data on position of individual fish fry was gathered every minute and presented as sum total distance travelled during dark periodes (left) and during light periods (right) in large movements (>6.1 mm, top), small movements (2.1-6.1 mm, middle) and inactivity (minor movements <2.1 mm, buttom). Data is presented as mean ± SD based on 4 replicates. Each replicate consisted of 4 individually exposed fry, or 8 for controls. Significant diffrences (p<0.05) compared to control are marked with *.

Highlights:

- Toxicity of CuO NPs and Cu ions varied among different zebrafish model systems.
- Very limited toxicity of either Cu form was detected in studies with ZFL cells.
- Overall, Cu ions were slightly or considerable more toxic than CuO NPs to both embryos and fry.